# THE EFFECT OF THE COPULATORY PLUG
# IN THE FUNNEL-WEB SPIDER, *AGELENA LIMBATA*
# (ARANEAE: AGELENIDAE)

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ABSTRACT. Some females of the funnel-web spider, *Agelena limbata* multiply mate. After copulation, males make a visible copulatory plug which covers the female's genital opening. I assessed the effect of copulatory plugs on the fertilization success of second males, by conducting double mating experiments, using fertile and sterilized males in sequence. When females copulated with only one fertile male, more than 90% of their eggs were fertilized. Some males deposited complete plugs and others incomplete plugs. The relative size of the males to females and absolute male size affected the completeness of plugs. Complete plugs prevented another male's insemination completely, but incomplete plugs allowed insemination by second males. In general, first males had higher fertilization success than second or later males, and copulatory plugs enhanced the first male's advantage.

In many taxa, after mating, the male deposits a copulatory plug that is thought to prevent intromission by other males. This is known in insects (Drummond 1984; Matsumoto & Suzuki 1992), mammals (Martan & Shepherd 1976), snakes (Devine 1975), ticks (Oliver 1974) and spiders (Levi 1959; Jackson 1980; Robinson 1982) .

In spiders, there are several reports on the presence of an amorphous secretion-like material blocking the epigynum of just-mated females functioning to prevent intromission by a second male. Other mechanisms for preventing fertilization by second males include the breaking off of the embolus tip in the female's genitalia (*Argiope*, *Nephila*), or the sticking of the cap that normally cloaks the virgin male's embolus tip in some Araneidae (Lopez 1987). Copulatory plugs are considered an adaptive strategy in relation to paternity assurance in spiders (Austad 1982). However, plugs are generally not 100% effective in preventing further mating (Eberhard 1985). Jackson (1980) presented evidence that some secondary mates of females could remove the plug deposited by the first mate.

The funnel-web spider *Agelena limbata* is an annual species with a conduit spermatheca in which there are two separate genital openings: one for copulation and one for oviposition. Some females of *A. limbata* are polyandrous, and males secrete an amorphous material blocking the epigynum of just-mated females.

To understand the evolution of copulatory plugs, their effects must be assessed. In this paper, the effect of copulatory plugs on fertilization success in the funnel-web spider, *A. limbata* was examined by conducting double fertilization experiments, in which one male was sterilized by exposure to $\gamma$-rays of Co60.

## METHODS

**The spiders.**—The funnel web spider *A. limbata* is an annual spider commonly distributed in Japan. Females deposit one or two egg sacs under their webs in September after the males disappear, and they protect the sacs for a few weeks before death. The web of this species consists of a flat sheet with an attached funnel extending into twigs of the surrounding vegetation. The sheet has no adhesive properties. After the final molt, the male spider leaves his web and starts his search for mates. Courtship behavior and copulation are performed in the daytime; the duration of copulation is about thee hours (193 $\pm$63 min; mean $\pm$S.D.) (Masumoto 1991).

**Field observations.**—Observation of the mating behavior of *A. limbata* was conducted from July to September, 1988 and 1989 at the Kyushu University campus, located east of Fukuoka city, Japan. Webs in the study area, located on trees less than 2 m above the ground, were marked and observed daily. In 1988, each web was visited daily in a predetermined order at 3 h intervals from 0900 to 2100 h. In 1989, each web

was visited daily at 1 h intervals from 0900 to 1500 h. Unmarked spiders found in the study area were captured whenever possible. Each spider was brought to the laboratory, anesthetized with $CO_2$ gas, and individually marked on the dorsal surface of the abdomen with a color marking pen. Within the day, the marked spiders were brought back to the entrance of their original web after another anesthetization. Spiders cohabiting with mates were not disturbed so as not to disrupt their mating behaviors. After the mating season from 1988 to 1992, females were collected to determine the condition of any copulatory plugs.

**Laboratory experiments.**—Sub-adult spiders were collected in June and July in "Aburayama Observation Park of Nature", Fukuoka, Japan. They were reared in plastic boxes (32 cm × 19 cm or 19 cm × 11 cm). Day length and temperature were almost similar to field conditions. Bees were given to females as a food every four days, a mealworm was supplied to males every week, and water was sprayed on both sexes every four days.

After spiders matured, I measured the cephalothorax width of individuals. Mating experiments were conducted in the morning because in this species mating is diurnal (Masumoto 1991). All experiments were started at between 0800–1000 h and lasted for at least six hours. Each individual was used only one time a day. Individual males chosen at random were released into a box containing a virgin female. After copulation, the genitalia of the females were examined to determine the condition of the copulatory plug. For some females, new males were introduced every day until a secondary copulation occurred.

To determine the fertilization success of secondary mates, double fertilization experiments were conducted using sequential matings of sterilized and fertile (non-treated) males. Males were collected in the field while immature and, after maturation, sterilized by exposure to 7 krad γ-ray of Co60. The behavior of sterile males did not seem to differ from that of unsterile males. Sterlized males were allowed to mate with virgin females, and after copulation occurred, the shape of copulatory plug was recorded. In some cases, after successful mating with a sterile male, a fertile (untreated) male randomly chosen was allowed to mate with the same female. In these cases, males were replaced everyday until the
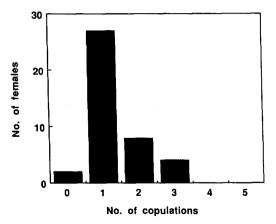


Figure 1.—The distribution of the total number of copulations of marked females through the mating season in 1989 and 1990.

second copulation occurred. The reverse experiment was also conducted with sequential matings of fertile (non-treated) and sterilized males. Females started to deposit their eggs in late August. In late September, the number of eggs and juveniles was counted. Values are represented as means ± SE.

## RESULTS

**Field observations.**—To assess the number of males which courted or copulated with a female, I used data on 41 females which were monitored from virginity to the end of mating season in each year. The end of mating season means the date when the last copulation in field was observed in each year (23 August in 1988 and 21 August in 1989). Females were courted an average of $3.76 \pm 2.12$ times [$4.10 \pm 0.37$ ($n = 29$) in 1988, $2.92 \pm 0.66$ ($n = 12$) in 1989] and copulated an average of $1.34 \pm 0.73$ times [$1.24 \pm 0.13$ ($n = 29$) in 1988, $1.58 \pm 0.23$ in 1989 ($n = 12$)]. Of 39 females which copulated, 12 (30.8%) copulated with an additional male (Fig. 1).

**Variation of copulatory plugs.**—During the first half of copulation, the male used only one of the palps for insemination. After this, he changed the position of the female which was laying on the web, and he used another palp to inseminate during the last half of copulation. After copulation, all males made a copulatory plug which was secreted from the palps. Some plugs were classified as complete; these covered the female genital opening completely. Others were classified as incomplete; these covered only a portion of

Table 1.—Mean cephalothorax width of males and females, mean ratio of male cephalothorax width to female cephalothorax width, and mean age of males when he first copulated with a virgin female according to condition of copulatory plugs. Values are means $\pm$ SE. * Mann-Whitney $U$ test.

|  | Complete plug | Incomplete plug | $z$* | $P$* |
|---|---|---|---|---|
| $n$ | 31 | 19 |  |  |
| % | 62.0% | 38.0% |  |  |
| Mean cephalothorax width of males (mm) | 4.87 $\pm$ 0.06 | 4.67 $\pm$ 0.09 | 2.08 | 0.038 |
| Mean cephalothorax width of females (mm) | 4.79 $\pm$ 0.08 | 4.91 $\pm$ 0.09 | 1.02 | 0.307 |
| Mean ratio of male cephalothorax width/ female cephalothorax width | 1.02 $\pm$ 0.02 | 0.96 $\pm$ 0.02 | 2.06 | 0.039 |
| Mean age of males (days) | 15.4 $\pm$ 0.7 | 14.0 $\pm$ 1.1 | 1.53 | 0.126 |

the female genital opening. Among 50 untreated virgin males, 31 (62.0%) made complete plugs and 19 (38.0%) of them made incomplete plugs on the first copulation with virgin females.

To assess the cause of variation of the copulatory plugs, I analyzed the relation between the classification of copulatory plugs and relative male size to female size (male cephalothorax width/female cephalothorax width), maximum cephalothorax width of males and females, and male age (Table 1). The mean cephalothorax width of males, and the ratio of male to female cephalothorax width, was greater in males that made complete plugs than in males that made incomplete plugs.

To evaluate the variation of plugs in field, I collected 20 females who were protecting their egg sacs at the end of the mating season. Of 20 females collected, 13 (65%) had complete copulatory plugs in their genitalia and the other 7 (35%) had incomplete plugs.

**Sperm usage pattern.**—Most eggs of singly-mated female hatched, and the proportion of eggs hatched was not different between females with complete and incomplete plugs. Most sperm were

sterilized by the radiation treatment because no eggs hatched when females copulated with sterile males. I could not obtain data about fertile male/sterile male double copulations. When plugs were complete, only the first male's sperm were used in fertilizing eggs. But when plugs were incomplete, the second male's sperm were used for fertilizing 62.9 % of eggs (Table 2).

From the observation of 19 cases of pre-copulatory behavior with non-virgin females (in 10 cases, complete plugs; in 9 cases, incomplete plugs), I found that before insemination of females with incomplete plugs, five second males removed the copulatory plug deposited by previous males, but that four second males did not remove the copulatory plug which was too small to cover the epyginum. Males hooked incomplete plugs with the palp and pulled the plug out. But no male could pull out any complete plug. After removal of an incomplete copulatory plug, males started insemination and covered the genital opening of the female with a new plug after insemination. In seven females which initially had incomplete plugs and then copulated with another male, five of them received complete

Table 2.—Proportion of hatched eggs per female in the experiments with sequential mating of sterilized and fertile males in the spider *Agelena limbata*. Sample number is indicated in parentheses. Values are means $\pm$ SE. * Mann-Whitney $U$ test.

| Mating regime | | Proportion hatched | | | |
|---|---|---|---|---|---|
| First male | Second male | Complete plug | Incomplete plug | $U$* | $P$* |
| Fertile | — | 94.0 $\pm$ 5.3% (14) | 90.0 $\pm$ 9.1% (5) | 37 | ns |
| Sterile | — | 0.0 $\pm$ 0.0% (3) | 0.0 $\pm$ 0.0% (3) |  |  |
| Fertile | fertile | 88.1 $\pm$ 11.9% (4) | 93.0 $\pm$ 7.1% (4) | 8.5 | ns |
| Sterile | fertile | 0.0 $\pm$ 0.0% (4) | 62.9 $\pm$ 11.4% (6) | 24 | <0.01 |
| Fertile | sterile | 100.0 $\pm$ 0.0% (4) | — |  |  |

plugs in their genitalia after the second mating. Plugs did not come out by themselves even after oviposition.

## DISCUSSION

From data obtained in field, about 70% of females copulated with a male only once. So first males had a mating advantage compared to the second or later males. But 30.8% of females remated with the next courting male in the field. The reason why some females copulate more than once is unknown in *A. limbata*. Watson (1991b) hypothesized that in the sierra dome spider, *Linyphia litigiosa*, the second mating is important for females as bet-hedging against a cryptic or unexpressed deleterious character present in the first male's genes. Re-mating with another male may be advantageous for some females in *A. limbata*.

There are no externally visible plugs in *Linyphia litigiosa* (Watson 1991a) and in *Frontinella pyramitela* (Austad 1982). In *L. litigiosa*, Watson (1991a) suggested that internal plugs would have to be quite subtle and sections of epigyna have not revealed any plugs, and that fertilization success of some secondary mates is high, although on average first mate sperm priority exists.

Females of *A. limbata* have a conduit spermatheca in which there are separate openings for entry and departure of sperm on opposite sites of the spermatheca. A conduit system has been thought to encourage a 'first-in/first-out' bias in sperm precedence favoring first males (Austad 1984; Watson 1991a). But when second copulations of females with incomplete plugs were successful, the proportion of eggs fertilized by the first male was reduced: second mates were able to fertilize 62.9% of eggs in the double fertilization experiments. Thus in *A. limbata*, though the mechanism of sperm competition of this spider is unknown, first male sperm precedence does not occur despite females having a conduit spermatheca; and gross spermathecal morphology is inadequate to explain sperm priority patterns as Watson (1991a) suggested.

When first mates do not make a complete copulatory plug, their fertilization success is reduced. Thus in *A. limbata*, the copulatory plug is very important in assuring first male sperm advantage. Incomplete plugs tended to be made by relatively small males, though the statistic was marginally significant. Smaller males may be unable to fill the genital opening of females with a plug secretion, but the precise cause of incompleteness of the copulatory plug in *A. limbata* is unclear.

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